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TECHNICAL MANUSCRIPT 296

IN VITRO INTERACTIONS BETWEEN RABBIT ALVEOLAR MACROPHAGES AND PASTEURELLA TULARENSIS

John E. Nutter
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MAY 1966

UNITED STATES ARMY
BIOLOGICAL CENTER
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IN VITRO INTERACTIONS BETWEEN RABBIT ALVEOLAR MACROPHAGES AND PASTEURELLA TULARENSIS

John E. Nutter

Quentin N. Myrvik

Medical Bacteriology Department
BIOLOGICAL SCIENCES LABORATORY

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In conducting the research reported here, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

FOREWORD

The material in this manuscript was submitted by John E. Nutter in partial fulfillment of the requirements for the Ph. D. degree to the Department of Microbiology, University of Virginia, Charlottesville. This work was partially supported by Research Grant AI-05667 from the National Institutes of Health, and a research grant from the North Carolina Tuberculosis Association. Quentin N. Myrvik is chairman of the Department of Microbiology, The Bowman Gray School of Medicine, Winston-Salem, North Carolina.

ABSTRACT

Rabbit alveolar macrophages were successfully employed in a study of host cell - Pasteurella tularensis interactions in vitro. Under cell culture conditions in which inhibitory antibiotics were not employed and small infection ratios were used, the relative in vivo virulence of two strains of P. tularensis was duplicated. As a consequence of intracellular multiplication, normal macrophages were killed in relation to the virulence of the strain employed. Alveolar macrophages were also collected from immune rabbits and macrophage mortality and bacterial growth were significantly suppressed below levels observed with normal macrophage preparations. The effect of immune serum could only be ascribed a minor role in the observed reactions. A marked intravenous toxicity of P. tularensis for the rabbit was observed with both virulent and attenuated strains. The toxicity was possessed only by viable preparations and could be elicited in animals immune to virulent challenge.

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I. INTRODUCTION

The results of several investigations demonstrated that classical circulating antibody plays at best only a minor role in the mechanisms of acquired resistance to Pasteurella tularensis.[1-5] It is necessary, therefore, to ascribe major importance to cell-associated factors when considering the mechanism of immunity to tularemia.

It was reported that peritoneal macrophages obtained from immune white rats had an enhanced ability to ingest P. tularensis in comparison with phagocytes obtained from normal animals. In addition, the immune macrophage system exerted a bacteriostatic effect on the intracellular organisms.[6] Similar findings were reported with rabbit and guinea pig macrophages.[4,5]

Additional evidence for the active participation of macrophages in the immune response was obtained in passive transfer studies.[7-9] These studies demonstrated the capacity of macrophages obtained from immune animals to confer on normal recipients increased resistance to challenge. The immunity possessed by recipients was proportional to the number of immune cells they received but was not correlated with the administration of immune serum. In addition, transfers between inbred animals resulted in a longer lasting immunity than those made between genetically dissimilar individuals.[7]

The potential problems associated with irritant-induced cells are exemplified by the work of investigators who demonstrated that when irritants were employed to stimulate rat peritoneal exudates, the results obtained following infection of these cells varied with the different eliciting agents.[10] Similar results were obtained with other experimental models.[11,12]

Pulmonary alveolar macrophages can be procured without an eliciting substance and thereby represent a population of phagocytic cells that closely approximate the normal in vivo physiological state.[13] The respiratory route has been utilized extensively for tularemic challenge and, in addition, this route has been employed successfully as a means of vaccinating monkeys and man.[14-16] The respiratory bronchioles were the primary sites of lodgement in the lungs of monkeys where the bacteria were shown to be within macrophages. Therefore, an opportunity existed for the study of the early stages of pulmonary tularemic infection.

The objectives of this study were to develop an in vitro model for studying intracellular infections and to determine by this means any differences in the response of macrophages from susceptible and immune hosts to P. tularensis.

II. MATERIALS AND METHODS

A. EXPERIMENTAL ANIMALS

New Zealand white rabbits weighing between 1.8 and 2.5 kg were used throughout the investigation.

B. TECHNIQUE FOR PROCURING ALVEOLAR MACROPHAGES

Rabbits were sacrificed by an air embolus or with an overdose of Pentothal sodium. The thoracic cavity was opened, the upper part of the trachea clamped shut with a hemostat, and the lungs insufflated with four 25-ml aliquots of Hanks balanced salt solution (BSS). BSS was introduced and withdrawn with a syringe and needle following puncture of the tracheal wall. The procedure described by Myrvik[13] was also employed with one modification, namely, the addition of 100 units of penicillin per ml of BSS.

C. VITAL STAINING

The viability of macrophages was determined by employing a trypan blue exclusion technique. For the test 0.2 ml of a macrophage suspension in the culture medium was mixed with 0.1 ml of 0.5% trypan blue. Macrophage suspensions were examined within 15 minutes as "wet mounts" in a standard hemacytometer and cells that excluded the dye were considered viable.

D. TISSUE CULTURE

Medium 199, pH 7.2 to 7.4, with 100 units of penicillin per ml and 20% pooled rabbit serum was employed for maintenance of macrophages. Two-ml aliquots of suspensions containing 2×10^6 macrophages per ml were distributed into 25-mm-diameter screw-cap test tubes and incubated at 36 C in an atmosphere of 5% CO_2 in air.

E. BACTERIAL STRAINS EMPLOYED

Strain SCHU S4 of *F. tularensis* is a fully virulent North American strain with a dermal LD_{50} for mice, guinea pigs, and rabbits of fewer than 10 organisms. The live vaccine strain (LVS) is a stable colonial variant derived from a heterogeneous Soviet vaccine strain and serially passed in white mice to increase its virulence and immunogenicity.[23] SCHU S1-11 is an attenuated strain similar in many characteristics to LVS but more virulent for the guinea pig.

F. BACTERIAL CULTIVATION

Liquid cultures were prepared in peptone cysteine broth (PCB) as described by Snyder et al.[17] Inocula for the PCB cultures consisted of 5% by volume of a culture grown in a modified casein hydrolyzate medium similar to that described by Mills et al.[18] PCB cultures were incubated at 37 C for 12 to 16 hr with agitation. Cultures were also prepared on glucose cysteine blood agar (GCBA) with incubation for 12 to 16 hr at 37 C in screw-cap tubes. GCBA was prepared from a dehydrated medium obtained from the Baltimore Biological Laboratories. Organisms were suspended in gel-saline diluent (0.85% sodium chloride and 0.1% gelatin) and adjusted to the desired concentration turbidimetrically. Stock cultures maintained on GCBA were stored at 5 C and transferred every 4 weeks.

G. BACTERIAL ENUMERATION

Viable numbers of P. tularensis were estimated by cultivation of appropriate dilutions on GCBA medium. Dilutions were prepared in gel-saline diluent and 0.1- to 0.2-ml portions were spread over the surface of the medium with a separate sterile glass rod for each plate. Replicate plates were prepared for each dilution and viable counts were estimated from the average number of colonies after 72 to 96 hours' incubation at 37 C.

H. MACROPHAGE INFECTION

Bacterial suspensions were diluted with medium 199 to contain the desired number of bacteria in the 0.1-ml inoculum routinely employed for each tube.

I. VACCINATION WITH LIVE TULAREMIA VACCINE (LVS)

Rabbits were vaccinated with 1.0×10^5 or 1.0×10^8 viable cells of LVS, employing a single subcutaneous injection in the flank of the animal with the organisms contained in 0.2 ml of gel-saline diluent. Animals were also vaccinated by placing a drop of a suspension containing 1.0×10^8 viable LVS cells per ml on the flank and utilizing the acupuncture technique for inoculation.

J. IMMUNIZATION TECHNIQUE EMPLOYING STRAIN SCHU S4

Rabbits were infected by the subcutaneous route with 1.0×10^3 cells of strain SCHU S4. The infection was allowed to proceed until the rabbits had been febrile (>103 F) for 24 hr. At that time, usually 4 days after infection, the animals each received 200 mg of streptomycin sulfate intramuscularly on 5 successive days, followed by 100 mg daily for 5 additional days.

K. SEROLOGY

Rabbits were bled from the marginal ear vein or by cardiac puncture at the time of sacrifice and agglutinin tests were performed with the sera according to the technique of Brigham.[19]

L. CHALLENGE

Animals were challenged with 1.0×10^8 cells of SCHU S4 by the subcutaneous route, observed daily for 30 days, and the survival intervals recorded.

III. RESULTS

A. QUALITY OF MACROPHAGES

The sources of rabbits were carefully screened to avoid colonies with a high incidence of chronic pulmonary infections due to members of the genus Bordetella.[20] More than 95% of macrophages collected from acceptable rabbits were viable, and uninfected control cultures survived in cell culture for up to 7 days.

B. EFFECT OF STREPTOMYCIN

During early studies to determine the feasibility of employing alveolar macrophages to investigate *F. tularensis* infections in vitro, both penicillin and streptomycin were included in the tissue culture medium. At a ratio of 10 bacteria per macrophage, the findings were the same as those with non-infected control cultures, indicating an inhibitory action of the antibiotics. The results of further experiments are presented in Figure 1. Normal macrophages were infected at a ratio of 1:1 with strain SCHU S4; 2 hours later streptomycin was added so that the concentrations indicated were achieved. Concentrations of 100, 50, and 10 micrograms per ml of streptomycin were lethal at both 24 and 48 hr, but one microgram per ml gave equivocal results. In addition, comparable results were obtained when experiments were performed with EVS. The inhibitory effect of streptomycin on intracellular *F. tularensis* was further evidenced by a decreased percentage of dead macrophages at the sampling intervals, reflecting the reduction in bacterial growth within the macrophages.

Subsequent experiments were performed with only penicillin added to the culture medium. As anticipated, penicillin did not affect the intracellular growth of *F. tularensis* in alveolar macrophages.

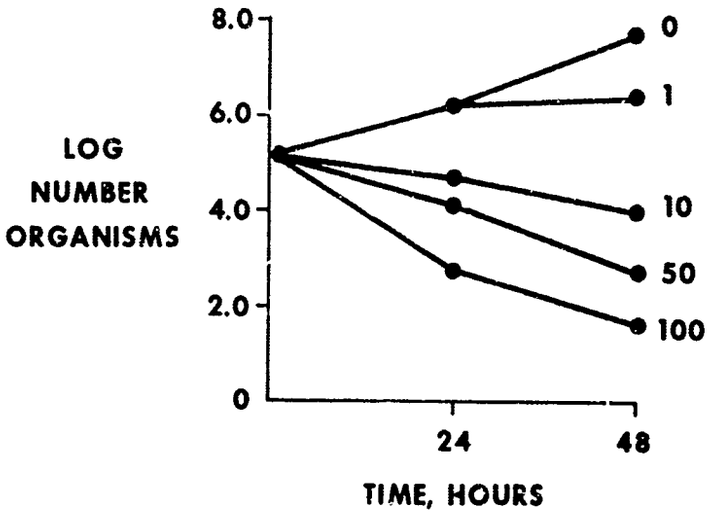

Figure 1. Effect of Various Concentrations of Streptomycin on the Intracellular Viability of Streptomycin-Sensitive Pasteurella tularensis. Streptomycin was added to the culture medium 2 hours after alveolar macrophages were infected at a ratio of one bacterium per macrophage. Numbers at right indicate the micrograms of streptomycin per milliliter of culture medium.

C. RESPONSE OF NORMAL ALVEOLAR MACROPHAGES TO INFECTION

The mortality response of alveolar macrophages following infection with 8 to 14 organisms per alveolar macrophage is shown in Figure 2. Each point indicates the mean value of replicate experiments and, at the 36- and 48-hr sampling times, the range of one standard deviation is presented. At 36 hr, 77% of the macrophages infected with fully virulent organisms were nonviable. Those cells infected with LVS exhibited a 25% mortality, but uninfected control cultures exhibited only a 3% mortality. Similar ratios were observed at 48 hours; however, the percentage of nonviable macrophages had increased. The presence of viable bacteria was required for the induction of the macrophage mortality response. Preparations containing heat-killed SCHU S4 produced no increase in mortality over that present in uninfected control cultures. The mortality response of normal alveolar macrophages, infected in vitro, reflected the response generally observed in the intact animal. Fully virulent strains of P. tularensis repeatedly caused the death of a greater percentage of alveolar macrophages than that caused by the attenuated live vaccine strain at comparable intervals.

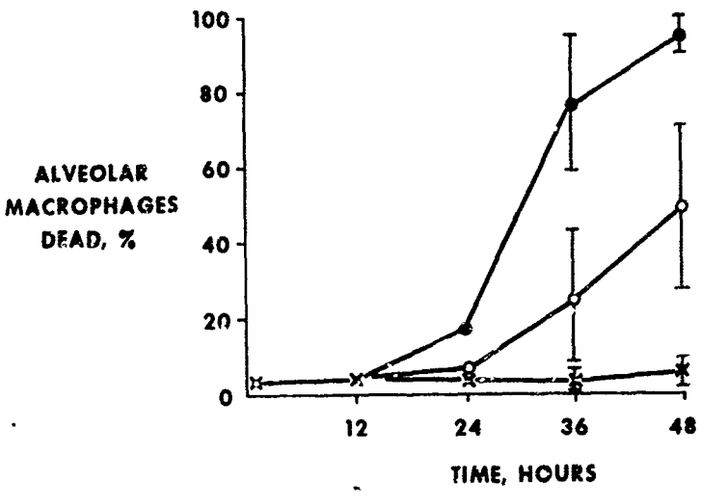

Figure 2. The in vitro Mortality Response of Alveolar Macrophages Following Infection with Pasteurella tularensis. Virulent strain SCHU S4, ●; live vaccine strain, LVS, O; uninfected control, X.

D. IMMUNIZATION STUDIES

The mortality response of rabbits vaccinated with LVS and subsequently challenged with strain SCHU S4 is presented in Table 1. The administration of LVS by acupuncture or by the subcutaneous route failed to engender significant protection against a subcutaneous SCHU S4 challenge. Although a slight increase in the survival time of the vaccinated groups was evident, all vaccinated animals succumbed to an acute progressive infection not unlike that seen in control animals. Table 1 also contains the results of immunizing rabbits by the SCHU S4-streptomycin treatment technique. All rabbits that had recovered from a previous virulent infection with the aid of streptomycin therapy survived the challenge with strain SCHU S4. The immunity produced by the SCHU S4-streptomycin procedure persisted for at least 120 days and was demonstrated in other studies when the animals were challenged with SCHU S4 contained in a small-particle aerosol.

TABLE 1. MORTALITY RESPONSE OF VACCINATED RABBITS FOLLOWING SUBCUTANEOUS CHALLENGE WITH PASTEURELLA TULARENSIS SCHU S4

Vaccination Procedure	No. Dead/ No. Challenged[a]	Average Day of Death (range)
1.0×10^9 LVS by acupuncture	5/5	8.6 (6-12)
1.0×10^9 LVS subcutaneously	5/5	8.2 (6-10)
1.0×10^5 LVS subcutaneously	5/5	7.6 (7-9)
SCHU S4-streptomycin 30 days prior to challenge	0/5	-
SCHU S4-streptomycin 120 days prior to challenge	0/5	-
Nonvaccinated	10/10	5.6 (4-8)

a. Challenge dose, 1.0×10^3 organisms.

Highest agglutinin titers were observed 2 weeks following initiation of the infection among SCHU S4-streptomycin treated animals with an average peak titer of 1:1280. Subcutaneous vaccination with 1.0×10^5 LVS cells caused only a minimal agglutinin response with an average peak titer of 1:7. Vaccination with 1.0×10^9 viable LVS organisms by the subcutaneous route or by acupuncture produced average peak titers of 1:416 and 1:120 respectively 1 week after vaccination.

One possible reason for the failure of LVS to immunize by the routes employed could be the inability to become disseminated from the original sites. A technique employed for overcoming this was the intravenous injection of large numbers of bacteria; the results are presented in Table 2. The injection of 1.0×10^{10} to 1.0×10^{11} viable attenuated organisms consistently caused rapid death of the rabbits. When 1.0×10^6 viable organisms of the virulent SCHU S4 strain were injected and streptomycin therapy was started 2 hours later, death resulted but was somewhat delayed. Injection of 1.0×10^9 viable LVS cells followed by streptomycin therapy did not result in the death of any of the test animals. When cells of the virulent strain were killed by formalin, their lethal action was abolished, even when the dose was increased 10 times the LD_{100} of living organisms.

TABLE 2. INTRAVENOUS TOXICITY OF PASTEURELLA TULARENSIS PREPARATIONS FOR TP⁻ RABBIT

Preparation	No. Dead/ No. Treated	Average Time of Death, hours
1.0×10^{10} viable LVS	5/6	9
1.0×10^{11} viable LVS	5/5	10
1.0×10^{11} viable SCHU S1-11	5/5	12
1.0×10^{9} viable SCHU S4 with streptomycin therapy	5/5	24
1.0×10^{9} viable LVS with streptomycin therapy	0/5	-
1.0×10^{10} formalinized SCHU S4	0/2	-
1.0×10^{11} formalinized SCHU S4	0/2	-
1.0×10^{10} viable LVS injected into immune animals	6/7	10

It was impossible to immunize rabbits by the intravenous injection of large numbers of attenuated organisms because of the lethal nature of this procedure. The precise mechanism of this rapid death is unknown. At necropsy, pathological findings consisted of extensive diffuse hemorrhagic lesions in the lungs and thymus gland. Microscopically, evidence of extravasated erythrocytes was also observed throughout most of the other tissues. Collectively, these observations suggest that the primary damage was localized in the vascular endothelium.

E. IN VITRO INFECTION STUDIES

Experiments were performed in which the bacteria:macrophage ratios were varied to increase the sensitivity of the assay system for detecting cellular immunity to P. tularensis. Macrophages were infected at 5 ratios ranging from 100 bacteria per macrophage to 1 bacterium per 100 macrophages, and the viability of the macrophages was determined daily for 3 days. The mortality response of the macrophages, with respect to per cent dead plotted against time, was proportional to the multiplicity of infection. Macrophage mortality was greater with the virulent organisms, but the response was still proportional to the bacteria:macrophage ratio. The relative virulence of LVS and SCHU S4 was readily discernible. The approximate ratios (bacteria:macrophage) required to kill 50% of the macrophages at 48 hr was 1:1 for LVS and only 1:10 for the virulent SCHU S4. When normal macrophages were infected with either the attenuated or virulent strain, maximum separation of values in response to the different ratios occurred 48 hr after infection.

When immune (SCHU S4-streptomycin) rabbits were used as a source of macrophages, the results of in vitro infections were markedly different. Forty-eight hours after infection, the initial LVS:macrophage ratio of 10:1 had produced a mortality of 62% of the immune macrophages compared with 90% in normal macrophages. Immune macrophages were almost completely resistant to LVS for 72 hr when 1:1 and 1:10 ratios were employed. In contrast, these ratios produced a mortality of 70 to 90% in normal macrophages at that time. Similar results were obtained with the virulent SCHU S4 organisms, although the degree of suppression was not as marked.

An inhibitory action of alveolar macrophages from immune rabbits on the intracellular growth of P. tularensis was also demonstrated. The interaction of normal and immune macrophages with LVS and strain SCHU S4, each at two multiplicities, is presented in graphic form in Figures 3 and 4. The figures contain the range of values as well as mean values for both macrophage mortality and viable bacterial counts. Infected immune suspensions consistently had fewer dead macrophages at 48 hours than did comparable populations of normal macrophages. The effectiveness of immune macrophages was also demonstrated by the differential in viable organisms present in the immune and normal macrophage cultures. Viable counts in the immune macrophage cultures were always 2 to 4 logs below those in normal macrophage cultures.

F. ROLE OF IMMUNE SERUM

When serum - medium 199 mixtures were inoculated with SCHU S4, the organisms multiplied slightly (<1 log) during the 48-hour observation period when either normal or immune serum was employed. In contrast, similar experiments with LVS showed that the viable count of this strain

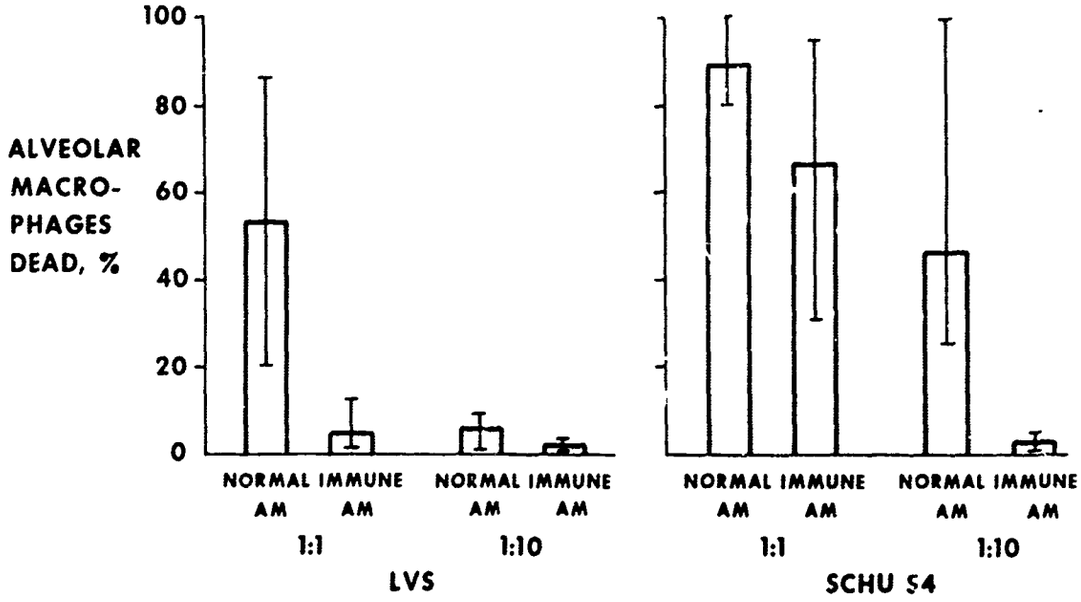

Figure 3. Alveolar Macrophage Mortality 48 Hours after in vitro Infection with Two Strains of Pasteurella tularensis each at Two Bacteria:Macrophage Ratios.

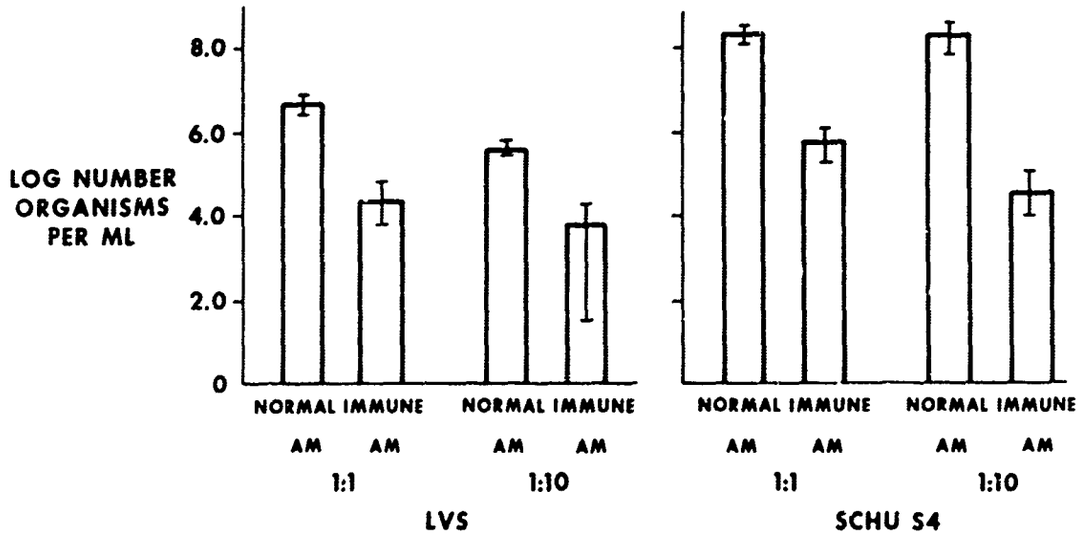

Figure 4. Viable Numbers of Two Strains of Pasteurella tularensis 48 Hours after in vitro Infection of Alveolar Macrophages at Two Bacteria:Macrophage Ratios.

decreased approximately 1 log during the same period in the presence of either normal or immune serum. These results indicate that immune serum did not exhibit enhanced bactericidal properties in comparison with normal serum. On the other hand, 20% serum in tissue culture medium 199 did not supply the cultural conditions necessary for optimum growth of either strain.

Experiments were also performed in which normal macrophages were suspended in medium 199 with immune serum and immune macrophages were suspended in medium 199 with normal serum. Results of an experiment with LVS, presented in Table 3, indicate that normal macrophages in concert with immune serum may be able to reduce the viable bacterial count. However, the reduction is less than that observed with immune macrophages suspended in immune serum and cannot account for the cellular immunity observed in this study. Immune macrophages suspended in normal serum were as active as the fully immune system, i.e., both macrophages and serum from SCHU S4-streptomycin treated rabbits.

TABLE 3. THE EFFECT OF DIFFERENT MACROPHAGE-SERUM COMBINATIONS ON THE GROWTH OF PASTEURELLA TULARENSIS LVS

Type of Macrophage	Type of Serum	Organisms per ml at 48 Hours[a]
Normal	Normal	1.8×10^5
Normal	Immune	7.5×10^4
Immune	Immune	1.8×10^3
Immune	Normal	3.8×10^3

a. 2.0×10^4 organisms per ml initially.

IV. DISCUSSION

The events that occur during the early stages of infection are difficult to study in the intact animal because the mass of the infecting organisms is usually relatively small. These events, however, probably determine whether or not the infecting agent can become established and cause overt disease. The availability of alveolar macrophages has provided an opportunity to study P. tularensis-macrophage relationships in vitro, simulating at least in part the in vivo conditions during the early postphagocytic period following aerosol challenge.

The observation that low levels of streptomycin were effective in inhibiting intracellular growth of P. tularensis in alveolar macrophages maintained in tissue culture is not in agreement with several reports in the literature and with usual experimental methods. Tularemia is classically an intracellular infection and in order for the drug to produce its rapid and profound effect in this disease, some of it must enter the cells that harbor organisms. Indeed, it is not surprising that streptomycin can enter metabolically stimulated alveolar macrophages. Normally, they are carrying out active pinocytosis and phagocytosis, which would allow extracellular components to enter nonspecifically the intracellular milieu. The findings by Hunt and Myrvik[21] that alveolar macrophages can take up appreciable quantities of preformed antibody illustrate that active uptake of even large protein molecules is apparently a normal process and strengthen this viewpoint.

The observation that live tularemia vaccine failed to immunize rabbits against challenge with fully virulent P. tularensis is in marked contrast to the results obtained with other animals. The mouse and guinea pig[22] and the monkey[23] as well as man[3,24,25] have responded to LVS vaccination with a high level of immunity. In this regard, it was shown in the present investigation that rabbits were capable of developing immunity to tularemic challenge, provided that they were infected with virulent organisms and then cured with streptomycin. Although LVS-vaccinated rabbits were not appreciably more resistant to challenge than nonvaccinated animals, they did produce agglutinins. The production of antibody without development of significant resistance to challenge is comparable to the course of events in other species following vaccination with killed tularemia vaccines.

During attempts to immunize rabbits with large numbers of P. tularensis a marked systemic toxicity was encountered. Moody and Downs[26] referred to this phenomenon in a study of the immunogenicity of various strains for the white mouse. They found that when mice were injected with living suspensions the majority of animals succumbed within 48 hours. In contrast, killed preparations failed to elicit the response and the toxicity resembled a rickettsial toxemia. We also found that killed suspensions failed to elicit toxic death. The observation that P. tularensis is capable of

producing lethal toxic reactions in the rabbit initially suggested that acquired immunity might involve antitoxic mechanisms. However, immunized rabbits were just as susceptible as normal rabbits to the toxic properties of viable suspensions. Clearly these data rule out antitoxic immunity as a dominant factor in acquired immunity to tularemia.

The data indicated that the mortality response of infected normal alveolar macrophages was proportional to the in vivo virulence of the strain of P. tularensis employed. Immune macrophages exhibited a lower mortality than normal macrophages when infected with either attenuated or fully virulent organisms. The validity of employing a decrease in macrophage mortality as a parameter of acquired immunity is supported by the evidence, which demonstrated an inhibition of bacterial growth by immune macrophages. Pulmonary alveolar macrophages collected from immune rabbits were more effective in suppressing the growth of P. tularensis than were equivalent numbers of normal macrophages. These observations established that acquired immunity to tularemia can be demonstrated in vitro employing alveolar macrophages maintained in a tissue culture system.

It was observed that immunity to P. tularensis, demonstrated in vivo, had no demonstrable antitoxic component. This same situation existed when macrophages were infected in vitro. Neither strain exerted an immediate toxic effect on normal macrophages but this effect followed intracellular multiplication of the organisms. The expression of immunity by macrophages from immunized animals was dependent on a suppression of intracellular bacterial growth preventing the intracellular population from exceeding the toxic threshold.

The results of supplying normal macrophages with immune serum yielded the same results in this study as reported by others.[4,5] Our studies showed that normal macrophages in concert with immune serum could limit the growth of LVS. However, the level of immunity was less than that of immune cells with either immune or normal serum. In contrast, immune macrophages suspended in normal serum did not demonstrate a diminution in activity. These results demonstrate the dominant role played by cell-associated factors in immunity to tularemia.

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Nutter, John E.
Myrvik, Quentin N.

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13. ABSTRACT

Rabbit alveolar macrophages were successfully employed in a study of host cell - Pasteurella tularensis interactions in vitro. Under cell culture conditions in which inhibitory antibiotics were not employed and small infection ratios were used, the relative in vivo virulence of two strains of P. tularensis was duplicated. As a consequence of intracellular multiplication, normal macrophages were killed in relation to the virulence of the strain employed. Alveolar macrophages were also collected from immune rabbits and macrophage mortality and bacterial growth were significantly suppressed below levels observed with normal macrophage preparations. The effect of immune serum could only be ascribed a minor role in the observed reactions. A marked intravenous toxicity of P. tularensis for the rabbit was observed with both virulent and attenuated strains. The toxicity was possessed only by viable preparations and could be elicited in animal immune to virulent challenge.

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